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ERBB2 is a target for USP8-mediated deubiquitination

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ABSTRACT

Overexpression and poor downregulation of ErbB receptor tyrosine kinases are associated with enhanced signaling and tumorigenesis. Attenuation of EGF-receptor (EGFR) signaling is mediated by endocytosis and ubiquitination by the E3-ligase Cbl. En route to lysosomes, but before incorporation of the EGFR into internal vesicles of MVBs, the EGFR undergoes Usp8-mediated deubiquitination. ErbB2 displays enhanced recycling back to the cell surface, and therefore we hypothesized that Usp8 is not part of the ErbB2 trafficking pathway. Here, we demonstrate, in the context of a chimeric EGFR-ErbB2 receptor, that (i) EGF induces pY1091 Cbl binding site-dependent K63-polyubiquitination of EGFR-ErbB2, (ii) Cbl is tyrosine phosphorylated upon stimulation of EGFR-ErbB2 wt and Y1091F mutant receptor, (iii) EGF-induced activation of EGFR-ErbB2 induces Usp8 tyrosine phosphorylation, and (iv) ubiquitination of the EGFR-ErbB2 wt and Y1091F mutant is enhanced upon coexpression of catalytically inactive Usp8-C748A in the presence and absence of EGF. We further show that Usp8 tyrosine phosphorylation upon stimulation of EGFR-ErbB2 is (a) independent of Y1091, (b) dependent on Src- and EGFR-ErbB2-kinase activity, (c) enhanced upon coexpression of Usp8-C748A, and (d) partly dependent on the Microtubule Interacting and Transport (MIT) domain of Usp8. Our findings demonstrate that Usp8 is part of the ErbB2 endosomal trafficking pathway.

1. Introduction

The ErbB family of receptor tyrosine kinases consists of four members denoted ErbB1 (also known as EGFR), ErbB2, ErbB3 and ErbB4, that form homo- and heterodimeric complexes upon ligand binding. ErbB2 is an orphan receptor, which functions by forming heterodimeric complexes with ligand-bound ErbB receptors, including the tyrosine kinase inactive ErbB3 [1]. The ErbB receptors are critical regulators of normal cellular growth, differentiation and tissue development in many different organs. While EGFR plays an essential role during epithelial-cell development [2,3], ErbB2 has been demonstrated to be important in the developing heart [4], central [5] and peripheral [6] nervous system and mammary gland morphogenesis [7]. Besides their role in normal physiology, EGFR, ErbB2 and ErbB3 are also frequently involved in the formation of solid tumors and as a consequence the ErbB signaling network is one of the main targets for anti-tumor therapy in humans [8]. ErbB2 overexpression triggers ligand-independent activation of the kinase domain and is observed in a large proportion of breast and ovarian cancers, where it is associated with tumour size, spread of the tumour, resistance to anti-oestrogen therapy and poor prognosis [9]. A humanized monoclonal antibody to the extracellular domain of ErbB2, Herceptin (trastuzumab), has been approved for clinical use [10].

Attenuation of ErbB receptor signaling is mediated by the clathrin-dependent pathway of receptor-mediated endocytosis and subsequent sorting of the activated ligand-receptor complex via the multivesicular body (MVB) pathway to lysosomes for degradation [11]. Thus, incorporation of ErbB receptors in internal vesicles of MVBs abrogates the communication with cytoplasmic signaling molecules, leading to attenuation of signaling. Moreover, uncontrolled ErbB receptor signaling that results from increased receptor expression, overexpression of ligands, or activating mutations in the transmembrane, tyrosine kinase or cytoplasmic domains is often correlated with decreased ErbB receptor downregulation (removal from the cell surface) [1].

Most studies on receptor downregulation have been performed on EGFR. Upon EGF binding, the Cbl family of RING finger E3-ubiquitin ligases catalyses the ligand-induced ubiquitination of the EGFR, which plays an important role in EGFR internalization and lysosomal targeting [11]. Cbl is recruited to the EGFR, either directly through its tyrosine kinase binding (TKB) domain to phosphorylated tyrosine residue pY1045 in the cytoplasmic tail of EGFR or indirectly via Grb2, which binds to phosphorylated tyrosine residues Y1068 and Y1086 [12]. The RING finger of Cbl simultaneously interacts with Ubc4/5 E2-ubiquitin activating enzymes that transfer the activated ubiquitin to lysine side chains of the EGFR substrate protein [13]. Ubiquitination can either exist as mono-, multi- (mono-ubiquitination on multiple lysines) or

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polyubiquitination. Whereas K48-linked polyubiquitination serves as a signal for proteasomal degradation, K63-linked polyubiquitination has been shown to regulate non-proteosomal processes such as DNA repair, kinase activation, translational regulation and vesicular trafficking [14]. Mono- and multi-ubiquitination has been implicated in ligand-mediated endocytosis [15,16]. Previous reports demonstrate that the EGFR is undergoing predominantly mono-, multi- and K63-linked polyubiquitination [17].

Much less is known about the role of ubiquitination in the intracellular routing of the other ErbB receptors. Previous studies have shown that ErbB2, ErbB3 and ErbB4 show impaired ligand-induced downregulation compared to EGFR [18,19]. On the other hand, ErbB2 and ErbB4 contain similar Cbl binding sites as EGFR [20–22]. The impaired downregulation of ErbB2 has been correlated with poor recruitment into clathrin-coated pits resulting in reduced internalization [18,19,23,24], impaired lysosomal targeting of the internalized receptor and enhanced recycling from sorting endosomes back to the plasma membrane [25–27]. Moreover, heterodimerization with ErbB2 impairs the downregulation of EGFR [25–28]. When comparing the molecular structure of EGFR and ErbB2, impaired downregulation of ErbB2 may result from (i) reduced recruitment of the clathrin adapter protein AP2μ to drive internalization [19,29,30], (ii) the absence of a dileucine signal in the cytoplasmic tail of ErbB2 that corresponds to EGFR LL1010/1011 which associates with AP2β to promote lysosomal targeting of the EGFR [22,31], (iii) the presence of a unique 45 amino-acid insert in the cytoplasmic tail of ErbB2 [32] and/or (iv) impaired recruitment of Cbl E3 ligases [33]. Notably, we previously demonstrated that the replacement of the EGFR Cbl binding site by that of ErbB2 did not affect Cbl recruitment, receptor-ubiquitination, -degradation, -downregulation or ligand degradation of EGFR, suggesting that poor downregulation of ErbB2 is not due to sequence variations in the Cbl binding site of these receptors [22].

We have previously demonstrated that, following ligand-induced Cbl-mediated ubiquitination, the EGFR is deubiquitinated before lysosomal degradation [34]. Indeed, two deubiquitinating (DUB) enzymes have now been demonstrated to deubiquitinate EGFR prior to incorporation into internal vesicles of MVBs [14]. AMSH is a metazoan-specific JAMM-type DUB that is specific for K63-linked polyubiquitin and has been proposed to limit sorting of the EGFR into the MVB pathway [35–37]. Usp8 is a member of the Ubiquitin specific protease (Usp) family that removes K48- and K63-linked polyubiquitin and has been proposed to recycle ubiquitin from substrate proteins to refill the cellular ubiquitin pool [38–43]. Both enzymes bind with their PxxP motif to the SH3 domain of the ESCRT-0 subunit STAM and with their N-terminal MIT domain to ESCRT-III subunits [14]. Using catalytically inactive dominant negative DUB enzymes, RNA interference techniques and gene knock-out models, it has been firmly established that AMSH and Usp8 deubiquitinate the EGFR [35–37,39–43]. While some reports suggest that Usp8 inhibits EGFR degradation [40], we and others demonstrated that Usp8 stimulates EGFR degradation [41,42]. Moreover, we also demonstrated that Usp8 is tyrosine phosphorylated in an EGFR- and Src kinase-dependent manner following EGF stimulation [41]. However, the functional significance of Usp8 tyrosine phosphorylation is as yet unknown.

Given the impaired downregulation of ErbB2, we have investigated in the present study whether ErbB2 and Usp8 functionally interact. Using a chimeric EGFR-ErbB2 model system, which can be activated by EGF and therefore allows a fair comparison between the EGFR and the orphan ErbB2 receptor, we demonstrate that ErbB2 is regulated by Cbl-mediated K63-linked polyubiquitination and Usp8-mediated deubiquitination, similarly to EGFR. Moreover, we show that Usp8 interacts with ErbB2 and is tyrosine phosphorylated in a ErbB2- and Src kinase-dependent manner, although its tyrosine phosphorylation is to a lesser extent than in the case of EGFR stimulation.

2. Material and methods

2.1. Reagents

The following antibodies were used in these studies: α-EGFR monoclonal antibody (mAb) 528, α-EGFR polyclonal antibodies (pAb) 1005, α-Cbl mAb C15, α-ErbB2 pAb C18, α-GFP pAb FL (Santa Cruz), α-EGFR mAb LA22, α-phosphotyrosine mAb 4G10, α-K63 ubiquitin mAb Apu3 (Upstate Biotechnology), α-FLAG mAb M2, α-ubiquitin pAb U5379 and α-tubulin (Sigma). Immunoprecipitation of EGFR was carried out using mAb528, while pAb1005 was used for EGFR detection on Western blots, unless specified otherwise. Goat anti-rabbit (GARPO) and goat anti-mouse (GAMPO) mAbs, both linked to horseradish peroxidase, were purchased from Signal Transduction Laboratories. Other reagents used in this study included Sepharose beads coupled to proteins A and G (Amersham Biosciences), Turbofect™ (Fermentas), EGF (BD Bioscience), Src Inhibitor I (Sigma) PP2 Src kinase inhibitor and PD153035 EGFR/ErbB2-tyrosine kinase inhibitor (Calbiochem). All PCR primers were obtained from Sigma.

2.2. Recombinant DNA technology and constructs

pcDNA3 EGFR and pcDNA3 EGFR Y1045F were kindly provided by Dr. Y. Yarden (Weizmann Institute, Israel). Mutations of the EGFR Cbl binding site were introduced as previously described by us [22]. The K721A mutation was introduced in pcDNA3 EGFR by PCR-mediated primer overlap extension using the *Eco81I* sites. pLTR2 EGFR-ErbB2^{IC} (denoted here as the EGFR-ErbB2 chimera) [18] was kindly provided by Dr. P.P. di Fiore (Fondazione Instituto FIRC di Oncologia Moleculare, Italy). The EGFR-ErbB2 was removed from the LTR2 vector by *SauI* and *XhoI* digestion, and subsequently inserted into *SauI–XhoI* digested pcDNA3 EGFR. The Y1091F mutation was introduced in pcDNA3 EGFR-ErbB2 chimera by PCR-mediated primer overlap extension using the *BstEII* and *XhoI* sites. The K733A mutation was introduced in pcDNA3 EGFR-ErbB2 by PCR-mediated primer overlap extension using the *Eco81I* and *BstEII* sites. pLXSN Neo was obtained from Dr. G.P. Nolan (Stanford University Medical Center) . The retroviral pLXSN EGFR construct has been described previously [22]. The EGFR and pcDNA3 EGFR-ErbB2 constructs containing mutations in the Cbl binding site were cloned into the modified pLXSN Neo vector using *VspI* and *XhoI* restriction sites. The pME18S Flag mUsp8 construct containing the Flag epitope-tagged murine Usp8 cDNA was kindly provided by Dr. N. Kitamura (Tokyo Institute of Technology, Japan) [44]. Construction of the catalytically inactive Flag mUsp8 C748A mutant has been described previously [41]. Both wild-type and C748A Flag mUsp8 cDNAs were subsequently cloned in frame to the C terminus of EGFP using the pEGFP C1 vector (Clontech), using *XhoI* and *MfeI* restriction sites. Construction of Usp8 Δ140 was performed by overlap extension PCR of pEGFP C1 Usp8 wt with a temporarily removed NotI site, and subsequent cloning using the *NotI* and *Acc65I* sites. Primer sequences are available upon request. All mutations were verified by DNA sequencing.

2.3. Cell lines and recombinant protein expression

Parental NIH3T3 cells, kindly provided by Dr. J. Schlessinger (Yale University, New Haven, CT), and human embryonic kidney cells (HEK293 cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS; Hyclone). Cells were trypsinized when confluent and seeded in flasks for regular maintenance. HEK293 cells were transiently transfected using Turbofect™ according to the manufacturer's protocol, while NIH3T3 cells were retrovirally infected with (mutant) EGFR or EGFR-ErbB2 constructs, as previously described for EGFR [22].

2.4. Cell lysis, immunoprecipitation, immunoblotting and densitometry

Cell lysis, immunoprecipitation, immunoblotting and densitometry were performed as described previously by us [22,34,41].

3. Results

3.1. Cbl-mediated ubiquitination of the ErbB2 cytoplasmic tail depends on Y1091

We previously demonstrated that the replacement of the EGFR Cbl binding site by that of ErbB2 did neither affect Cbl recruitment, receptor ubiquitination, degradation and downregulation, nor ligand degradation, suggesting that the poor downregulation of ErbB2 is not due to sequence variations in the Cbl binding site of these receptors [22]. Indeed, it has previously been demonstrated that Cbl is tyrosine phosphorylated and recruited to ErbB2 pY1091 [45]. Moreover, the ErbB2 Y1091F mutant showed decreased Cbl binding and ErbB2 ubiquitination [20]. However, little is known about ligand-induced ErbB2 ubiquitination. To test whether the Cbl binding site in ErbB2 is essential for Cbl recruitment, Cbl tyrosine phoshorylation and receptor ubiquitination, we made use of chimeric constructs containing the extracellular and transmembrane domains of the EGFR and the ErbB2 cytoplasmic tail. Retroviral cell lines containing such chimeric EGFR-ErbB2 receptors were generated and expression of chimeric receptors in retroviral cell lines was verified by Western blot (Fig. 1A, 4th panel).

To test whether Cbl induces ubiquitination of the EGFR-ErbB2 chimera, serum-starved cells were treated with EGF. As shown in Fig. 1A (upper panel), Cbl is tyrosine phosphorylated 15 min after EGF stimulation and coprecipitates with the tyrosine phosphorylated EGFR-ErbB2 chimera. These data indicate that Cbl is recruited to the EGFR-ErbB2 chimera upon stimulation, although less efficient than to EGFR itself (Fig. 1A, upper panel). Mutation of the Cbl binding site in the EGFR (Y1045F) and the chimeric EGFR-ErbB2 (Y1091F) reduced but did not completely abrogate EGF-induced Cbl tyrosine phosphorylation and coprecipitation of the ErbB receptor (Fig. 1A, upper panel). Moreover, as shown in Fig. 1B, the EGFR-ErbB2 chimera displayed prominent EGF-induced ubiquitination, while mutation of the ErbB2 Cbl binding site (Y1091F) in the context of the EGFR-ErbB2 chimera almost completely abolished EGF-induced ubiquitination of the ErbB2 cytoplasmic domain. In addition, using K63-linked polyubiquitin specific antibodies, we show that both EGFR and EGFR-ErbB2 undergo ligand-induced K63-linked polyubiquitination which is abolished in EGFR Y1045F and ErbB2 Y1091F mutant constructs (Fig. 1C). In contrast, we could not detect any ligand-induced increase in K48-linked polyubiquitination of either EGFR or EGFR-ErbB2 using K48-linked polyubiquitin specific antibodies (results not shown). These findings directly show the efficacy of the ErbB2 Cbl binding site in supporting ligand-induced ErbB2 K63-linked polyubiquitination, which agrees with our previous findings that the ErbB2 Cbl binding site is fully functional when introduced into EGFR [22].

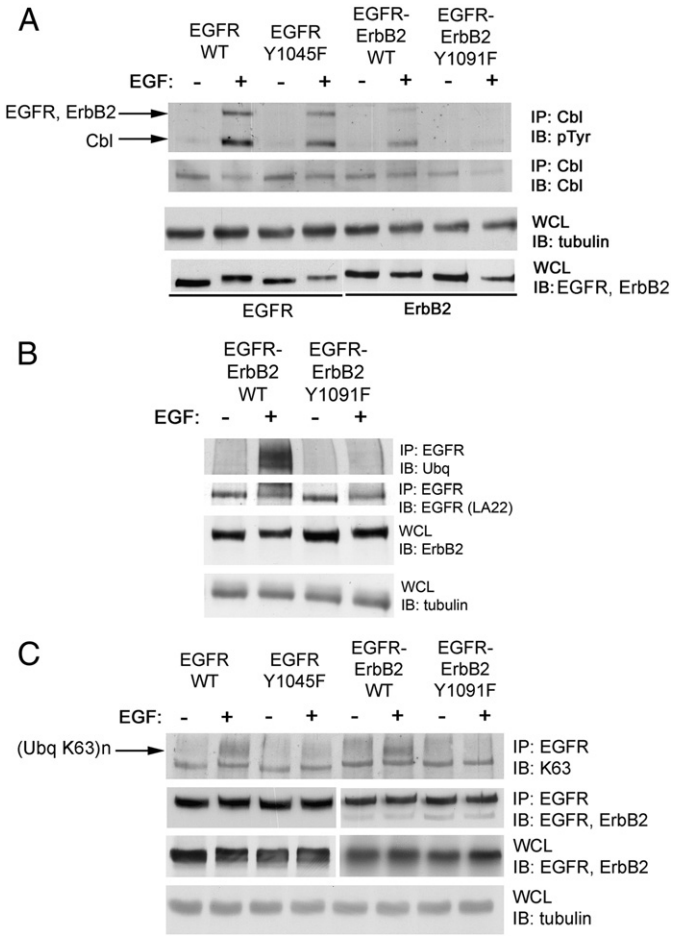

Fig. 1. Ligand stimulation couples EGFR-ErbB2 to Cbl-induced K63-linked polyubiquitination. NIH3T3 cells were retrovirally infected with EGFR wt, EGFR Y1045F, EGFR-ErbB2 or EGFR-ErbB2 Y1091F. A, Infected cells were serum starved overnight and stimulated for 15 min with 100 ng/ml EGF. Whole cell lysates (WCL) were used for anti-Cbl immunoprecipitation (IP) and immunoblot (IB) with the indicated antibodies. B, Infected cells were serum starved overnight and stimulated for 30 min with 100 ng/ml EGF. WCL were used for anti-EGFR IP and IB with the indicated antibodies. C, HEK293 cells were transiently transfected with EGFR wt, EGFR, Y1045F, EGFR-ErbB2 wt or EGFR-ErbB2 Y1091F. Serum-starved cells were EGF-stimulated for 15 min. WCL were used for anti-EGFR IP and IB with the indicated antibodies. Data are representative of 2–4 experiments.

3.2. Usp8 tyrosine phosphorylation upon stimulation of the chimeric EGFR-ErbB2 is enhanced in catalytically inactive Usp8 C748A mutant

The activity of E3 ligases is counterbalanced by the activity of deubiquitination enzymes. We previously reported that the deubiquitination enzyme Usp8 is tyrosine phosphorylated upon stimulation of EGFR [41]. However, Usp8 is present on endosomal membranes and since ErbB2 shows efficient recycling from early endosomes back to the cell surface [25], it is possible that internalized ErbB2 and Ups8 do not interact within the cell. To address whether Usp8 is tyrosine phosphorylated upon stimulation of EGFR-ErbB2, we co-transfected HEK293 cells with EGFR-ErbB2 and EGFP-Flag-Usp8. As shown in Fig. 2, Usp8 becomes tyrosine phosphorylated upon treatment of EGFR-ErbB2 with EGF, although to a lesser extent than in EGFR stimulated cells. Moreover, activated EGFR-ErbB2 coprecipitates with tyrosine-phosphorylated Flag-tagged Usp8, demonstrating that Usp8 binds to this chimeric receptor. This finding demonstrates that Usp8 is not only a substrate of ErbB2-induced tyrosine kinase activity, but also that Usp8 and ErbB2 colocalize inside the cell.

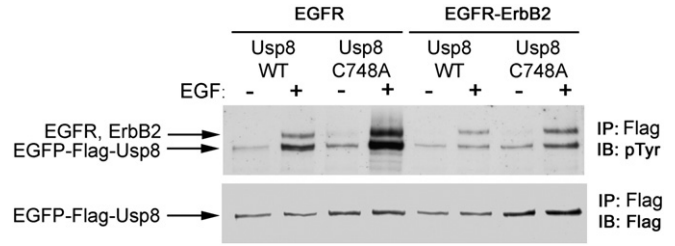

Fig. 2. Ligand stimulation of EGFR-ErbB2 induces Usp8 tyrosine phosphorylation. HEK293 cells were transiently transfected with EGFP-Flag-Usp8 wt or C748A mutant, as well as with EGFR or EGFR-ErbB2. Serum-starved cells were EGF-stimulated for 1 h and WCL were used for anti-flag IP and IB with anti-pTyr and anti-flag antibodies as indicated. Data are representative of 2 experiments.

It has previously been shown that a catalytically inactive mutant of Usp8 (C748A) displays enhanced binding to activated EGFR, which has been attributed to substrate-trapping on the endosomal membrane [40,46]. Consistent with this model, our data in Fig. 2 show that Usp8-C748A displays enhanced tyrosine phosphorylation and increased coprecipitation with the receptor upon EGF stimulation of both EGFR or EGFR-ErbB2 containing cells. These findings strongly suggest that the Usp8-C748A mutant shows enhanced steady state binding to both EGFR and EGFR-ErbB2 relative to Usp8 wild-type, which is consistent with the model that the C748A mutation interferes with dissociation of Ups8 from the ubiquitinated ErbB receptor.

3.3. Usp8 tyrosine phosphorylation is independent of an intact Cbl binding site

To determine whether Usp8 tyrosine phosphorylation is critically dependent on ErbB receptor ubiquitination by Cbl, we analyzed Usp8 tyrosine phosphorylation after EGF stimulation of cells containing the Cbl binding mutants EGFR-Y1045F and EGFR-ErbB2-Y1091F (Fig. 3A–D). Quantification of the Usp8 tyrosine phosphorylation signal of three independent experiments indicates that Usp8 wt and its C748A mutant were similarly tyrosine phosphorylated in cells with ErbB wild-type or the Cbl binding mutant receptors (Fig. 3C–D), although the level of Usp8-C748A tyrosine phosphorylation appeared somewhat decreased in the EGFR-Y1045F and EGFR-ErbB2-Y1091 mutants cells in the experiments shown in Fig. 3A–B. Also the extent of coprecipitation of in particular Usp8-C748A with the EGFR-Y1045F and EGFR-ErbB2-Y1091F mutant receptors was slightly decreased in this experiment compared to their wild-type counterparts, but again this was not confirmed by quantitative densitometric analysis of multiple experiments (data not shown). Collectively, these data demonstrate that the Cbl binding site of the wild-type EGFR and the EGFR-ErbB2 chimera is not required for efficient EGF-induced Usp8 tyrosine phosphorylation or coprecipitation of the ErbB receptors with Usp8.

3.4. Usp8 tyrosine phosphorylation upon stimulation of EGFR-ErbB2 is Src- and ErbB2- kinase dependent

The data presented in Figs. 2 and 3 suggest that the mechanism responsible for EGF-induced Usp8 tyrosine phosphorylation might be the same for EGFR and ErbB2. Our previous results have shown that Usp8 tyrosine phosphorylation upon EGF-induced stimulation is EGFR- and Src- tyrosine kinase dependent [41]. To test whether the kinase activity of ErbB2 is required for Usp8 tyrosine phosphorylation in EGFR-ErbB2 containing cells, we used the EGFR/ErbB2-kinase inhibitor PD153035. As shown in Fig. 4A–B (3rd panel), this inhibitor effectively blocked EGF-induced tyrosine phosphorylation of both EGFR and ErbB2. In agreement with our previous observation, Usp8 tyrosine phosphorylation following stimulation of EGFR was completely blocked in the presence of PD153035 (Fig. 4A, top panel, and 4C). In EGFR-ErbB2 expressing cells, EGF-induced Usp8 tyrosine phosphorylation was even lower in the presence of PD153035 than the Usp8 tyrosine phosphorylation level observed in unstimulated and mock-treated cells (Fig. 4B, top panel, and D), which is likely due to the fact that 18 h of serum-deprivation does not result in complete downregulation of ErbB2 kinase activity. As expected, coprecipitation of autophosphorylated EGFR and

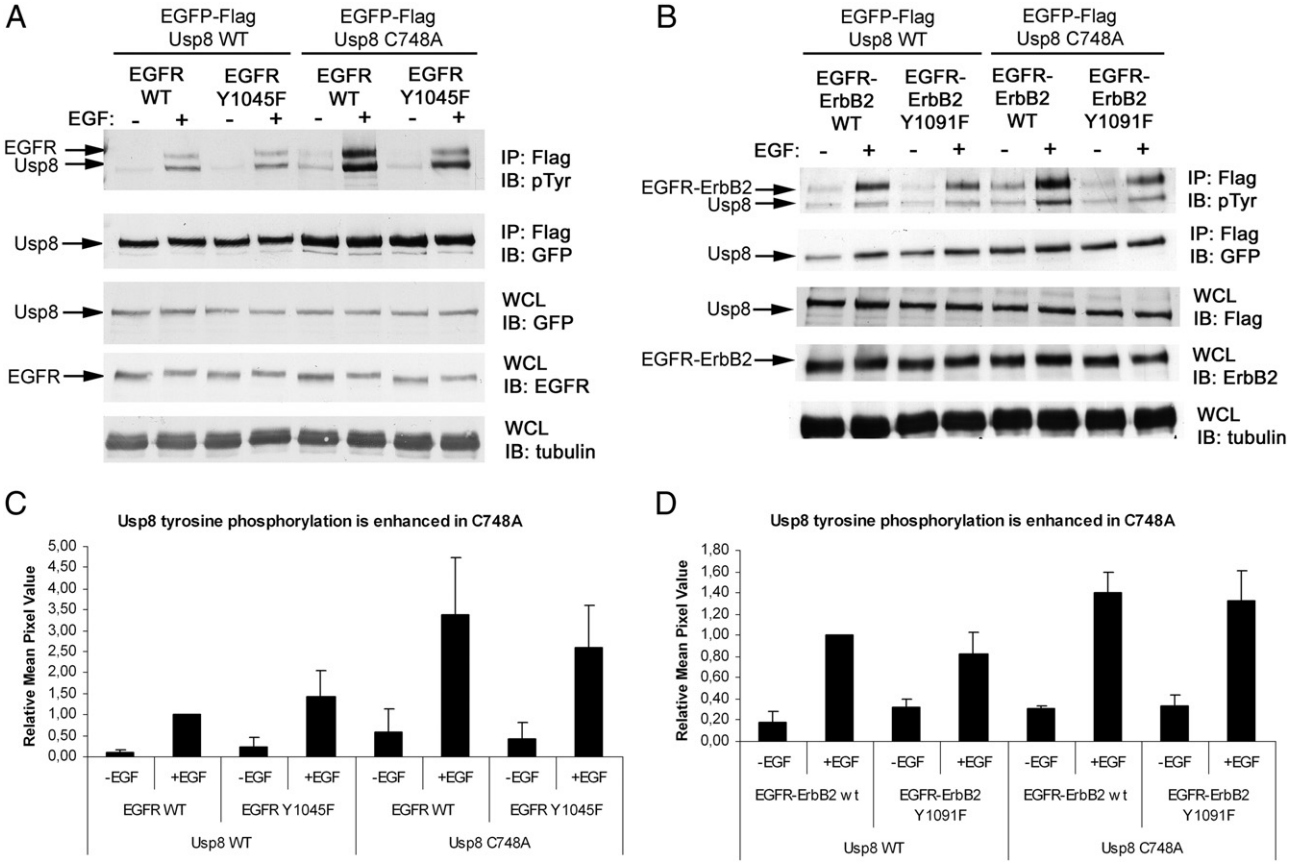

Fig. 3. Usp8 tyrosine phosphorylation is independent of an intact Cbl binding site. A and B, HEK293 cells were transfected with EGFP-flag-Usp8 wt or C748A mutant and with either EGFR wt, EGFR-Y1045F, EGFR-ErbB2 wt or EGFR-ErbB2 Y1091F constructs. Serum-starved cells were stimulated for 1 h with 100 ng/ml EGF after which WCLs were used for anti-flag IP and IB with the indicated antibodies. C and D, densitometric relative corrected mean pixel values of Usp8 tyrosine phosphorylation plotted as mean ± standard error of the mean (SEM). Phospho-signal of the IPs in A and B were related to the amount of precipitated Usp8 in the second panels and normalised to the EGFR/EGF or EGFR-ErbB2/EGF group. Data are representative of 3 experiments.

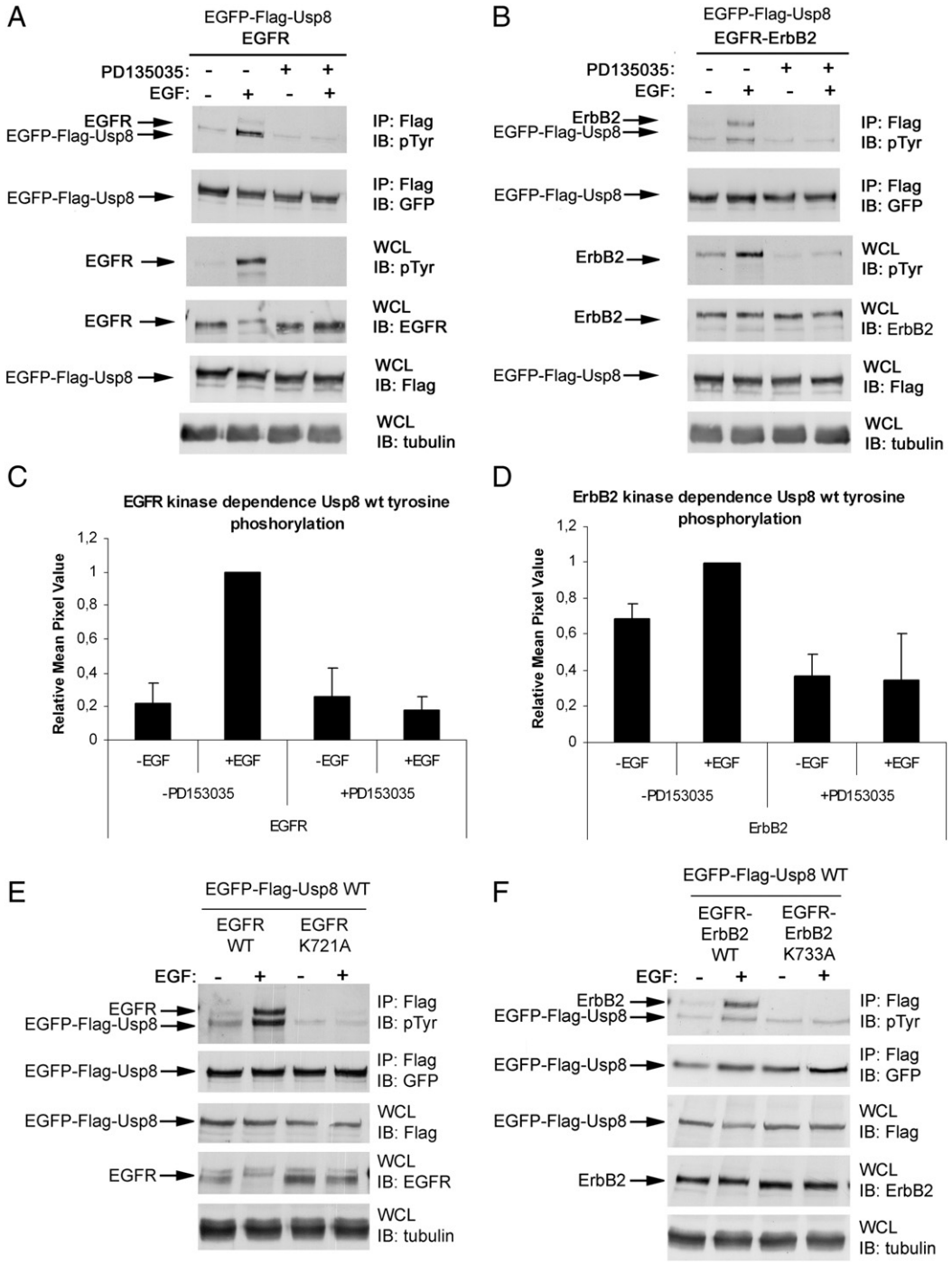

Fig. 4. Usp8 is tyrosine phosphorylated in an EGFR/ErbB2-kinase-dependent manner. A and B, HEK293 cells were transfected with EGFP-flag-Usp8 and either EGFR or EGFR-ErbB2. Serum-starved cells were pre-treated for 1 h with 10 μM PD153035 or DMSO and subsequently stimulated for 1 h with 100 ng/ml EGF. WCL were used for anti-flag IP and IB with the indicated antibodies. C and D, densitometric relative corrected mean pixel values of Usp8 tyrosine phosphorylation plotted as mean ± SEM. Phospho-signal of the IPs in A and B were related to the amount of precipitated Usp8 in the second panels and normalised to the EGFR/EGF or EGFR-ErbB2/EGF group. E and F, HEK293 cells were transfected with EGFP-flag-Usp8 and with either EGFR wt, EGFR K721A, EGFR-ErbB2 wt or EGFR-ErbB2 K733A. Serum-starved cells were stimulated for 1 h with 100 ng/ml EGF. WCL were used for anti-flag IP and IB with the indicated antibodies. Data are representative of 3 experiments.

EGFR-ErbB2 with Usp8 was no longer observed in the presence of the kinase inhibitor (Fig. 4A and B, top panel). To provide further evidence for ErbB kinase-dependent Usp8 tyrosine phosphorylation, we generated kinase-dead mutants of EGFR and EGFR-ErbB2. Stimulation of EGFR K721A and EGFR-ErbB2 K733A resulted in complete inhibition of Usp8 tyrosine phosphorylation and abrogated the coprecipitation of the autophosphorylated EGFR and EGFR-ErbB2 with Usp8 (Fig. 4E–F).

EGFR- and ErbB2-tyrosine kinases activate various downstream signaling molecules including members of the Src-family of tyrosine kinases [47]. To test whether Src-family tyrosine kinases may be responsible for Usp8 tyrosine phosphorylation upon stimulation of EGFR-ErbB2, we used the specific Src-family kinase inhibitors PP2 and Src Inhibitor I. Complete inhibition of Usp8 tyrosine phosphorylation in the presence of PP2 was achieved in both EGFR and EGFR-ErbB2 transfected cells (Fig. 5A–B, top panel, and Fig. 5C–D). To further support these results, we also used another inhibitor, Src Inhibitor I. As expected, Src Inhibitor I resulted in complete inhibition of Usp8 tyrosine phoshorylation upon EGFR stimulation, even though EGFR

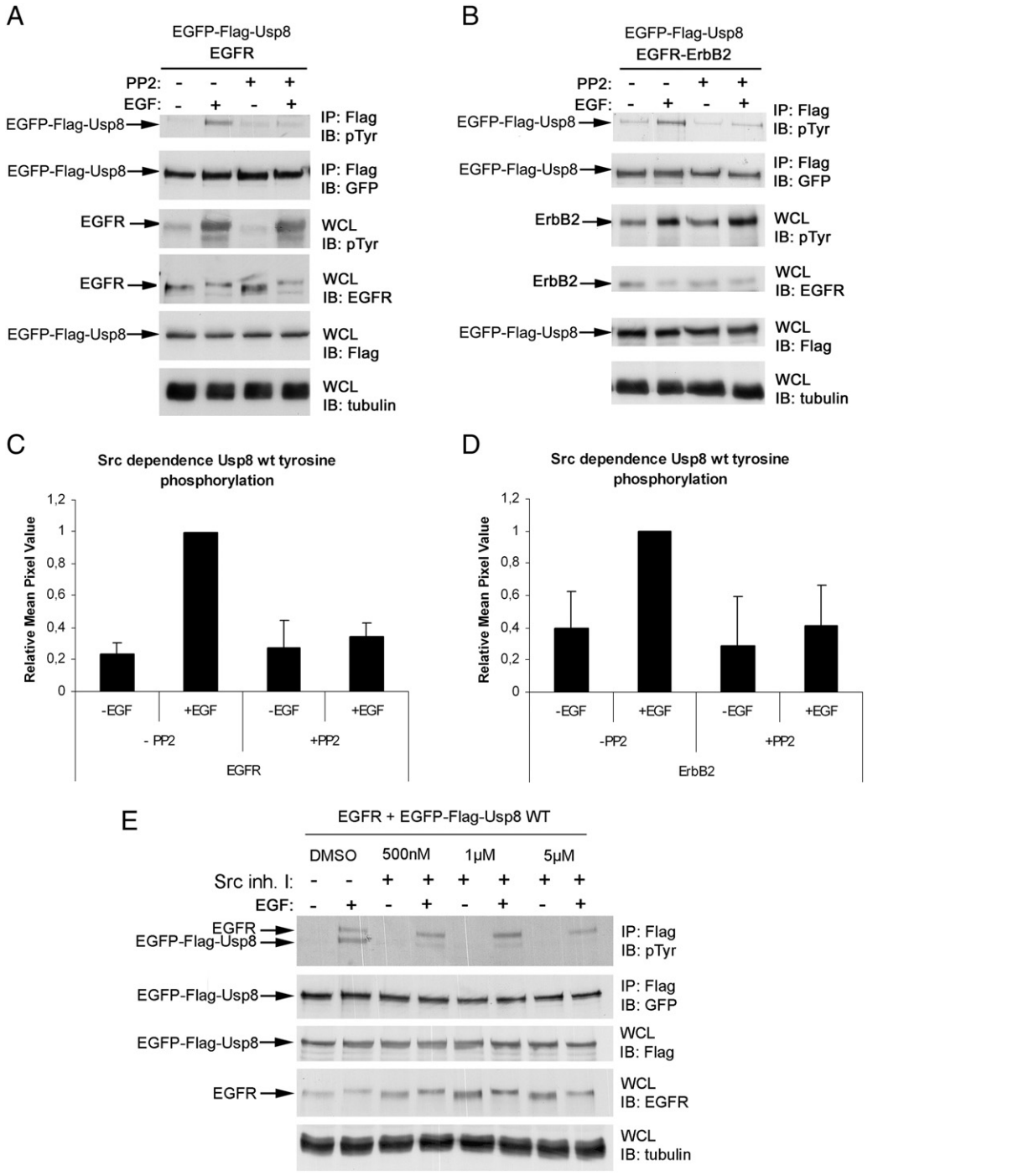

Fig. 5. Src kinase activity is essential for EGFR-ErbB2-mediated Usp8 tyrosine phosphorylation. A and B, HEK293 cells were transfected with EGFP-flag-Usp8 and either EGFR or EGFR-ErbB2. Serum-starved cells were pre-treated for 1 h with 5 μM PP2 or DMSO and subsequently stimulated for 1 h with 100 ng/ml EGF. WCL were used for anti-flag IP and IB with the indicated antibodies. C and D, densitometric relative corrected mean pixel values of Usp8 tyrosine phosphorylation plotted as mean + SEM. Phospho-signal of the IPs in A and B were related to the amount of precipitated Usp8 in the second panels and normalised to the EGFR/EGF or EGF-ErbB2/EGF group. E, HEK293 cells were transfected with EGFP-flag-Usp8 and EGFR wt. Serum-starved cells were pre-treated for 1 h with Src inhibitor I or DMSO and subsequently stimulated for 1 h with 100 ng/ml EGF. WCL were used for anti-flag IP and IB with the indicated antibodies. Data are representative of 3 experiments.

phosphorylation remained largely intact (Fig. 5E). These data extend our previous findings that Usp8 is tyrosine phosphorylated in an EGFR- and Src kinase-dependent manner [41] and demonstrate that Usp8 is a substrate for ErbB2-activated Src-family tyrosine kinases.

3.5. Optimal Usp8 tyrosine phosphorylation is MIT domain dependent

The MIT (Microtubule Interacting and Transport) domain, which is located at the N-terminus of Usp8, interacts with Escrt-III CHMP proteins and is important for endosomal recruitment [43]. Furthermore, the MIT domain of Usp8 is required for EGFR degradation [43], suggesting that this domain is essential for proper Usp8 function. Given that ErbB receptors are present on endosomal membranes, we hypothesized that MIT-dependent endosomal recruitment of Usp8 is required for Usp8 tyrosine phosporylation. In cells expressing either EGFR (Fig. 6A and C) or EGFR-ErbB2 (Fig. 6B and D), removal of the MIT domain (Usp8 Δ140) resulted in a decrease of the EGF-induced Usp8 tyrosine phosphorylation, when compared to Usp8 wt.

However, removal of the MIT domain did not completely abolish Usp8 tyrosine phosphorylation. Nevertheless, our findings clearly demonstrate that optimal Usp8 tyrosine phosphorylation requires an intact Usp8 MIT domain.

3.6. ErbB2 is undergoing deubiquitination by Usp8 in an EGF-dependent and -independent system

The findings described above demonstrate that Usp8 is part of the ErbB2 signaling cascade. We previously showed that overexpression of the Usp8-C748A mutant strongly enhances accumulation of the steady state level of ubiquitinated EGFR in the absence of EGF [41]. To provide evidence that EGFR and EGFR-ErbB2 are substrates for Usp8 deubiquitination activity, we performed an *in vivo* deubiquitination assay for which the EGFR wt, EGFR-Y1045F, EGFR-ErbB2 wt or EGFR-ErbB2-Y1091F receptors were co-transfected with either Usp8 wt or Usp8-C748A mutant into HEK293 cells. As expected, EGF stimulation of serum-starved cells resulted in an increased molecular size of EGFR as a result of ubiquitination, which was largely abolished in the EGFR Y1045F Cbl binding site mutant (Fig. 7, upper panel, 1st four lanes, arrow a). Interestingly, coexpression of Usp8-C748A with EGFR in unstimulated cells led to accumulation of ubiquitinated EGFR isoforms that migrated at a size above the 170 kDa EGFR band (Fig. 7, upper panel, 5th lane, arrow b), but clearly below the EGF-induced ubiquitinated forms of EGFR (arrow a). Moreover, in EGF-stimulated cells expressing both EGFR and Usp8 C748A, we observed not only a modest increase in the amount of high MW ubiquitinated EGFR species (Fig. 7, 6th lane, arrow a), but also a strong increase in the low MW ubiquitinated EGFR forms (arrow b). The low size ubiquitinated EGFR isoforms were also detected when Usp8-C748A was coexpressed with the EGFR-Y1045F Cbl binding site mutant (Fig. 7, 7th and 8th lanes, arrow b). It is possible that the former signal (arrow a) represents highly multi- or polyubiquitinated EGFR forms, whereas the latter signal (arrow b) represents mono- or oligo-ubiquitinated EGFR species.

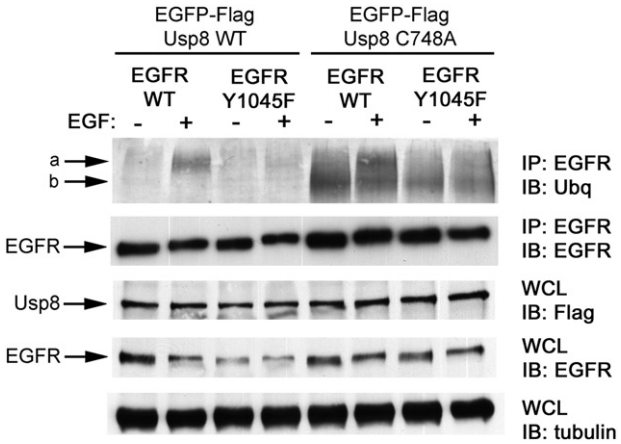

Fig. 7. Usp8 deubiquitinates EGFR by an EGF-dependent and -independent mechanism. HEK293 cells were co-transfected with EGFR wt or Y1045F mutant and either EGFP-flag-Usp8 wt or C748A mutant. Serum-starved cells were stimulated for 15 min with or without EGF. WCL were used for anti-EGFR IP and IB with the indicated antibodies. Data are representative of 3 experiments.

To determine whether these findings can be extended to the chimeric EGFR-ErbB2 model system, we performed similar experiments for EGFR-ErbB2 wt and EGFR-ErbB2-Y1091F. EGFR-ErbB2

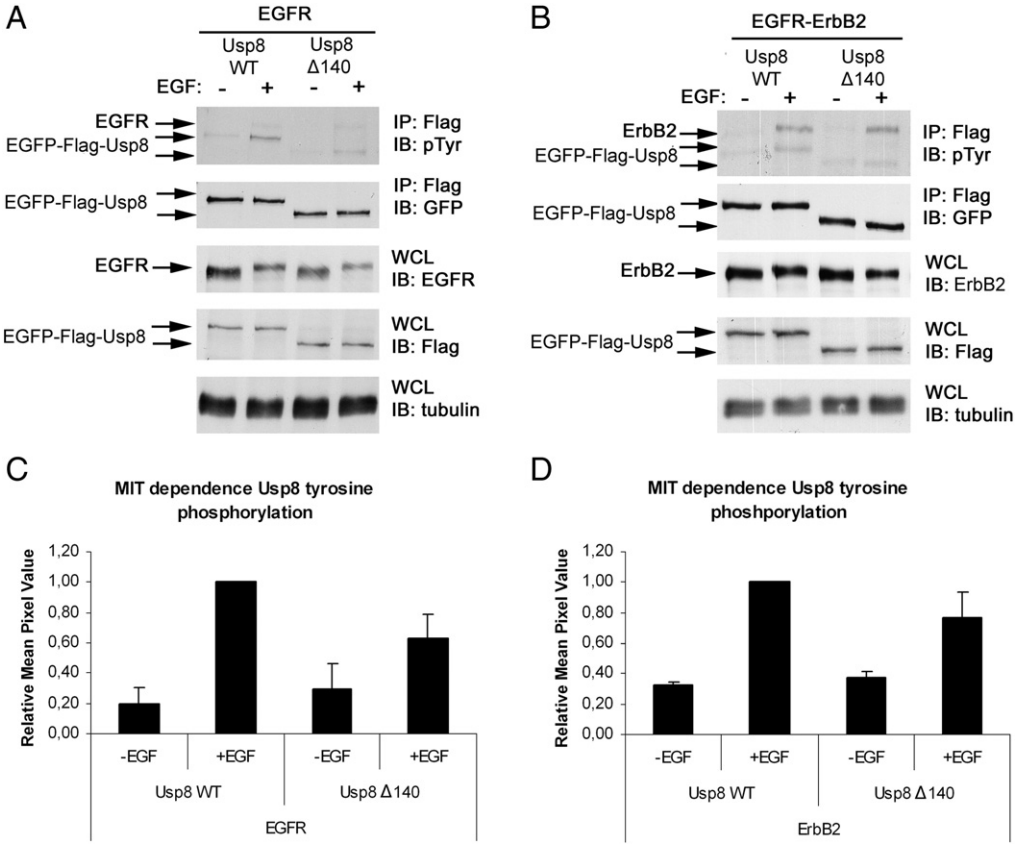

Fig. 6. The Usp8 MIT domain is required for optimal EGFR-ErbB2-induced Usp8 tyrosine phosphorylation. A and B, HEK293 cells were transfected with EGFP-flag-Usp8 wt or Δ140 and either EGFR or EGFR-ErbB2. Serum-starved cells were stimulated for 1 h with 100 ng/ml EGF. WCL were used for anti-flag IP and IB with the indicated antibodies. C and D, densitometric relative corrected mean pixel values of Usp8 tyrosine phosphorylation plotted as mean ± SEM. Phospho-signal of the IPs in A and B were related to the amount of precipitated Usp8 in the second panels and normalised to the EGFR/EGF or EGFR-ErbB2/EGF group. Data are representative of 3 experiments.

displayed EGF-induced ubiquitination resulting in a high molecular size band, while the EGFR-ErbB2- Y1091F Cbl binding site mutant showed almost no EGF-induced ubiquitination (Fig. 8, 1st four lanes, arrow a). Coexpression of Usp8-C748A with EGFR-ErbB2 wt only modestly decreased accumulation of the high molecular size ubiquitination signal induced by EGF stimulation (Fig. 8, 5th and 6th lanes, arrow a). This contrasts with the EGFR model system shown in Fig. 7, where expression of inactive Usp8 resulted in only a modest increase of high molecular size ubiquitination signal, which suggests that another enzyme may be involved in deubiquitinating ErbB2 (Fig. 8, arrow a). However, in cells coexpressing Usp8-C748A with EGFR-ErbB2 wt or EGFR-ErbB2-Y1091F a marked increase in the intensity of the low MW ubiquitination band was observed in both EGF-stimulated and -unstimulated cells (Fig. 8, 2nd four lanes, arrow b). Overall, these data demonstrate that ErbB2 is a substrate for the Usp8 deubiquitinating enzyme.

In conclusion, our findings demonstrate that Usp8 removes ubiquitin not only from the EGFR wt and Y1045F mutant receptor, but also from both the EGFR-ErbB2 wt and Y1091F mutant receptor in both ligand-independent and ligand-dependent model systems.

4. Discussion

The results presented in this study demonstrate that the deubiquitinating enzyme Usp8 is part of the ErbB2 endosomal trafficking pathway. In the context of a chimeric EGFR-ErbB2 receptor, we have shown that (i) EGF-induced K63-linked polyubiquitination of the ErbB2 cytoplasmic tail occurs efficiently in a pY1091-dependent manner, (ii) c-Cbl is efficiently tyrosine phosphorylated upon stimulation of the EGFR-ErbB2 wt and Y1091F mutant, (iii) EGF-induced activation of the EGFR-ErbB2 chimera induces Usp8 tyrosine phosphorylation, and (iv) ubiquitination of the EGFR-ErbB2 chimera (wt and Y1091F) is enhanced upon coexpression of catalytically inactive Usp8-C748A mutant in both an EGF-dependent and -independent system. We further show that Usp8 tyrosine phosphorylation upon stimulation of EGFR-ErbB2 is (a) independent of Y1091, (b) dependent on Src- and ErbB2- kinase activity, (c) enhanced upon coexpression of catalytically inactive Usp8 C748A, and (d) partly dependent on the MIT domain of Usp8. Our results are consistent with the model that ErbB2 is less efficiently sorted into the MVB pathway compared to the EGFR.

Here, we demonstrate that c-Cbl is tyrosine phosphorylated and recruited to the ligand stimulated chimeric EGFR-ErbB2 receptor, albeit to a lesser extent than the EGFR (Fig. 1). These results contrast with those of Levkowitz and coworkers, who initially reported that, using the same model system, Cbl is not tyrosine phosphorylated and recruited to ErbB2 [33], Subsequently, these authors demonstrated that Cbl is recruited to ErbB2 pY1091 of the oncogenic Neu ErbB2 receptor and in wt ErbB2 upon Herceptin-induced crosslinking [20,21,45]. We have previously reported that the Y1091 Cbl binding site of ErbB2 can functionally replace the EGFR Y1045 Cbl binding site without any obvious decrease in Cbl tyrosine phosphorylation, Cbl recruitment or receptor ubiquitination [22]. Overall, these findings suggest that the decreased efficiency of steady state Cbl tyrosine phosphorylation level and steady state Cbl recruitment to ErbB2 is not primarily due to the inefficiency of the ErbB2 Y1091 Cbl binding site, but rather a consequence of an as yet undefined mechanism that limits steady state recruitment of Cbl to the ErbB2 receptor. It is possible that decreased sorting of ErbB2 into the MVB pathway and/or enhanced recycling of ErbB2 back to the cell surface limits steady state Cbl-ErbB2 interaction, which may be caused by the presence or absence of a unique regulatory region in the ErbB2 cytoplasmic tail. As previously indicated, this may relate to (i) the absence of a dileucine signal in the cytoplasmic tail of ErbB2 at a position corresponding to EGFR LL1010/1011, which associates with AP2β to promote lysosomal targeting [22,31], and (ii) the presence of a unique 45 amino-acid insert in the cytoplasmic tail of ErbB2 that may be responsible for enhanced recycling [32]. Regardless of the exact mechanism, our results demonstrate that ligand stimulation couples ErbB2 to Cbl RING E3 ligase, resulting in receptor-induced ErbB2 ubiquitination (Fig. 1B). Furthermore, using K63-linked polyubiquitin specific antibodies, we show that EGFR and EGFR-ErbB2 undergo ligand-induced K63 polyubiquitination which is dependent on an intact Cbl binding site. This result is reminiscent of previous findings demonstrating that the EGFR is undergoing predominantly mono-, multi- and K63-linked polyubiquitination [17] and extends ligand-induced K63-linked polyubiquitination to ErbB2.

We also showed that Usp8 is coprecipitated and tyrosine phosphorylated upon EGF stimulation of the EGFR-ErbB2 chimera (Fig. 2). Interestingly, the Cbl binding site of EGFR-ErbB2 is not required for Usp8 tyrosine phosphorylation and coprecipitation (Fig. 3), suggesting that Usp8 might be recruited to the EGFR-ErbB2 also via mechanisms other than interaction between the Usp8 DUB domain and ubiquitinated cargo (e.g. via Hbp/STAM [44] or the MIT domain [43]). This finding extends our previous observations that Usp8 is tyrosine phosphorylated upon stimulation of the EGFR and that Usp8 is recruited to the EGFR via multiple interactions including (direct) binding of the Usp8 DUB domain to ubiquitinated EGFR and (most likely indirect) recruitment of Usp8 to the EGFR on endosomal membranes via the Usp8 N-terminal MIT domain [41].

Ligand-induced EGFR-ErbB2-mediated Usp8 tyrosine phosphorylation was lower than observed for EGFR. This may be explained by the fact that ErbB2 displays reduced internalization and enhanced recycling from early endosomes back to the cell surface when compared to the EGFR [18,19]. Furthermore, it has been shown that Usp8 localizes at early endosomes and removes ubiquitin from proteins after their sorting into the MVB pathway, but before incorporation of cargo proteins into internal vesicles of MVBs [14]. Thus, our results are consistent with the model that only a relatively small fraction of Usp8 is subjected to ErbB2-induced tyrosine phosphorylation because less ErbB2 receptors are sorted into the MVB pathway. Alternatively, the stoichiometry of ErbB2-induced Usp8 tyrosine phosphorylation may be lower than in the case of EGFR. It has been shown that the enzymatically inactive Usp8-C748A mutant displays substrate-trapping on the endosomal membrane [40,42] and consistent with this model, we demonstrated that Usp8 C748A displays enhanced tyrosine phosphorylation (Figs. 2 and 3). Overall, we showed that Usp8 is a substrate of ErbB2-induced tyrosine kinase activity.

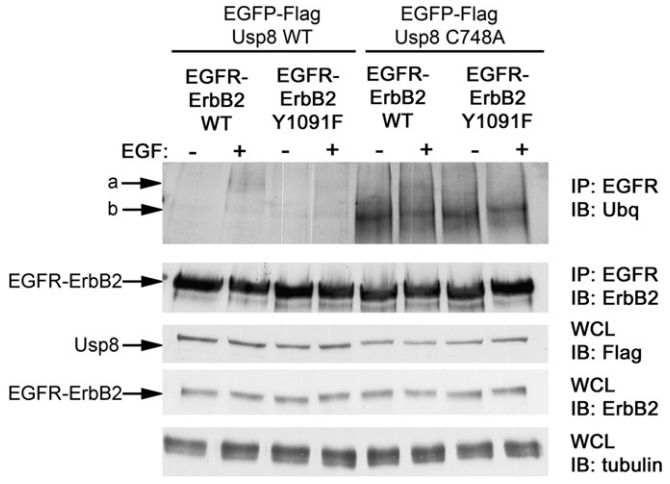

Fig. 8. Usp8 deubiquitinates EGFR-ErbB2 by an EGF-dependent and -independent mechanism. HEK293 cells were co-transfected with EGFR-ErbB2 wt or Y1091F mutant and either EGFP-flag-Usp8 wt or C748A mutant. Serum-starved cells were stimulated for 15 min with or without EGF. WCL were used for anti-EGFR IP and IB with the indicated antibodies. Data are representative of 3 experiments.

Our current findings further demonstrate that Usp8 binds to ErbB2 and that Usp8 tyrosine phosphorylation is dependent on ErbB2- (Fig. 4) and Src- kinase (Fig. 5) activities, thereby extending our initial findings in the EGFR model system to ErbB2 [41]. Src is an SH2- and SH3-domain containing cytoplasmic tyrosine kinase acting downstream of ErbB receptors, where it regulates a wide variety of cellular functions. It has been reported that interaction of tyrosine-phosphorylated ErbB2 in mammary tumors enhances Src kinase activity [48,49]. Moreover, using chimeric EGFR-ErbB2 receptors, it has also been shown that Src associates with ErbB2 [47,50]. We therefore hypothesize that tyrosine phosphorylation of Usp8 occurs in an ErbB receptor-Src-Usp8 trimolecular complex.

Our finding that the MIT domain of Usp8 is essential for optimal Usp8 tyrosine phosphorylation (Fig. 6) is consistent with the model that Usp8 is tyrosine phosphorylated upon MIT-dependent recruitment of Usp8 to endosomal membranes. The MIT domain in Usp8 was first identified by Row and coworkers, who showed that the MIT domain interacts with Escrt-III CHMP proteins and is necessary for the recruitment of Usp8 to endosomal membranes [43]. Although we cannot exclude the possibility that reduced Usp8 tyrosine phosphorylation upon removal of the MIT domain is due to conformational effects, we believe this explanation is less likely as this mutant has been extensively studied before [43]. Interestingly, removal of the MIT domain does not completely abolish Usp8 tyrosine phosphorylation, suggesting that Usp8 might be recruited to endosomal membranes also via alternative mechanisms (e.g. via Hbp/STAM [44] or ubiquitin [41]). Alternatively, the remaining tyrosine phosphorylation of the Usp8-Δ140 mutant may also indicate that Usp8 is partly phosphorylated by activated Src-family kinases in the cytoplasm. Finally, it should also be noted that at present we cannot rule out the possibility that Usp8 is tyrosine phosphorylated within its MIT domain. Nevertheless, our findings demonstrate that optimal Usp8 tyrosine phosphorylation requires an intact Usp8 MIT domain, which is consistent with the proposed role of the MIT domain in recruitment of Usp8 to endosomal membranes where activated ErbB receptor-Src complexes are located.

Most importantly, our current findings not only demonstrate that Usp8 is a substrate for ErbB2-induced tyrosine kinase activity but also that ErbB2 is a substrate for Usp8-mediated deubiquitination (Fig. 8). Moreover, overexpression of dominant negative Usp8-C748A led to enhanced ubiquitination of EGFR and EGFR-ErbB2 under both ligand-dependent and -independent conditions (Figs. 7 and 8). These findings indicate that EGFR and EGFR-ErbB2 are constitutively ubiquitinated and deubiquitinated. The Cbl binding site mutants of EGFR (Y1045F) and EGFR-ErbB2 (Y1091F) are also deubiquitinated by Usp8 both with and without EGF stimulation (Figs. 7 and 8). We therefore propose that Usp8 is not solely counteracting Cbl-mediated ubiquitination but also counteracts ligand- and Cbl-independent ErbB receptor ubiquitination. Our findings suggest that Usp8 mainly removes the low molecular size ubiquitination signal from ErbB receptors and we suggest the possibility that these signals represent mono- or oligo- ubiquitinated ErbB receptor isoforms (Figs. 7 and 8, upper panel, arrow b). This hypothesis is supported by our finding that EGFR wt and EGFR-ErbB2 wt but not their Y1045F and Y1091F Cbl binding site mutants undergo ligand-induced K63-linked polyubiquitination (Fig. 1). Indeed, the Y1045F and Y1091F Cbl binding site mutants are deubiquitinated by Usp8, suggesting that this ubiquitination signal may represent mono- or oligo-ubiquitin (Figs. 7 and 8, upper panel, arrow b). However, additional studies are required to verify this hypothesis. Indeed, it has previously been suggested that Usp8 may disassemble mono- and oligo-ubiquitin chains, in addition to K63- and K48-linked chains [40,42]. Thus, the role of Usp8 in the removal of monoubiquitin adducts has not yet been confirmed, neither *in vitro* nor *in vivo*. Our results further suggest that E3 ligases other than Cbl may be involved in ligand-independent low molecular size ubiquitination of both EGFR and EGFR-ErbB2. In addition, when overexpressing inactive Usp8 together with EGFR-ErbB2, a slight decrease in high molecular size ubiquitin was detected. It is possible that other DUBs may also be involved in deubiquitination of ErbB2.

5. Conclusions

The results presented in this study demonstrate that (i) Usp8 is a substrate for ErbB2- and Src-induced tyrosine kinase activity, (ii) EGF stimulation of the EGFR-ErbB2 chimera results in pY1091 Cbl binding site-dependent K63-linked polyubiquitination of the ErbB2 cytoplasmic tail, and that (iii) ErbB2 is a substrate for Usp8-mediated deubiquitination.

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